MOLECULAR AND CELLULAR BIOLOGY, June 1982, p. 607–616
0270-7306/82/060607-10$02.00/0

Vol. 2, No. 6

Phage Particle-Mediated Gene Transfer to Cultured Mammalian Cells

MASAHIRO ISHIURA,[1]* SUSUMU HIROSE,[2] TSUYOSHI UCHIDA,[1,3] YOSHIO HAMADA,[2] YOSHIAKI SUZUKI,[2] AND YOSHIO OKADA[1,3]

Department of Cell Biology[1] and Department of Developmental Biology,[2] National Institute for Basic Biology, Myodaiji, Okazaki 444, and Department of Animal Virology, Research Institute for Microbial Diseases, Yamada-oka, Suita 565,[3] Japan

Received 13 November 1981/Accepted 15 February 1982

Recombinant phage particles carrying the thymidine kinase (TK) gene of herpes simplex virus type 1, coprecipitated with calcium phosphate, efficiently transformed mouse Ltk$^-$ cells to the TK$^+$ phenotype. The conditions necessary to achieve high efficiency of transfer of the TK gene by phage particle-mediated gene transfer were investigated. Of the parameters examined, the pH of the buffer used for coprecipitation of phage particles with calcium phosphate, the length of time of coprecipitation, and the length of the adsorption period were found to alter the transfer efficiency significantly. The optimal pH was 6.87 at 25°C. The other optimal values for these parameters were as follows: coprecipitation time, 7 to 20 min; adsorption time, 18 to 30 h. Treatment with dimethyl sulfoxide, glycerol, or sucrose did not enhance gene transfer. The optimal conditions yielded about 1 transformant per 10^5 phage particles per 10^6 cells without carrier DNA. An increase in the dosage of phage particles, up to at least 5×10^7 phage particles per 100-mm dish, resulted in a linear increase in the number of transformants. Addition of carrier phage, up to 10^{10} phage particles per dish, did not significantly affect the number of transformants.

Recently, two selectable marker genes, the avian thymidine kinase (TK) gene (29) and the Chinese hamster adenine phosphoribosyl transferase (APRT) gene (20), have been isolated from mammalian cells by biochemical transformation of mutant cells with genomic DNA from wild-type cells which had been ligated with pBR322 marker sequences functional in *Escherichia coli* before application to the mutant cells. For application of this procedure in cloning of other selectable marker genes, however, several improvements are necessary, because the efficiency of transformation by genomic DNA is not high enough to overcome the spontaneous backmutation of the mutant cells (38, 39). The transformants obtained by DNA transfer are unstable (17), and the DNA sequences introduced exogenously are rearranged (20). The rearrangement of introduced DNA sequences greatly hinders the rescuing of a selected gene together with a marker sequence functional in *E. coli*.

Metaphase chromosomes have also been used for transfer of single-copy genes in eucaryotes (2, 17, 24, 26, 42) and have at least two advantages over genomic DNA as donors: the efficiency of gene transfer is always 10 to 100 times more than that of genomic DNA, and the transformants are more stable than those obtained by genomic DNA transfer (17). These advantages, we think, are partially due to the organization of the chromosome structure, where DNA sequences are covered with nuclear proteins, packaged compactly into the chromatin structure, and protected from attack by DNase. In spite of these advantages, however, metaphase chromosomes are not ideal donors for isolation of a selectable marker gene, because the DNA sequences in chromosomes cannot be manipulated before application to the recipient cells.

Therefore, we used phage particles as donors of genetic materials for the following reasons. (i) Phage DNA sequences are covered with phage coat proteins, packaged compactly into phage particles, and protected against attack from DNase. From this structure, we would expect a high efficiency of gene transfer and great stability of the resultant transformants when phage particles are applied to recipient cells. (ii) The method for construction of the gene library of mammalian DNA in lambda phage vectors has been established (1, 10, 16, 22, 33), and sequences of genomic DNA and lambda vectors can be manipulated easily before packaging into phage particles.

In this work, phage particles coprecipitated with calcium phosphate were applied to recipient cells for gene transfer in place of either DNA or chromosomes. Using Ltk$^-$ cells and Charon

4A recombinant phage carrying the TK gene of herpes simplex virus type 1 (HSV-1), we established conditions for phage-mediated gene transfer and attained a high efficiency of gene transfer. On the basis of these observations, we discuss possible applications of phage-mediated gene transfer.

MATERIALS AND METHODS

Cell culture. Bromodeoxyuridine-resistant mouse L cells deficient in TK activity (27) were maintained in Stanners modified Eagle minimum essential medium (α-MEM) (32) supplemented with 10% calf serum. Rat F2408 cells deficient in TK activity were kindly provided by A. Hakura and also maintained in α-MEM.

Enzymes. Restriction endonucleases were obtained from New England Biolabs or Takara Co. Digestion conditions were as recommended by the suppliers. T4 DNA ligase was prepared as described previously (37).

Plasmid DNA. The HSV-1 TK gene in the 2.0-kilobase (kb) *Pvu*II fragment was cloned into the *Pvu*II site of pBR322 (pTK4). A pTK4 derivative that carries a 7.6-kb *Eco*RI/*Hin*dIII fragment of fibroin DNA (35), pTK4miniFb, was kindly provided by Y. Tsujimoto.

Plasmid DNA was prepared from chloramphenicol-treated cells (3) by the cleared-lysate technique and purified by Sepharose 2B gel filtration and subsequent cesium chloride-ethidium bromide centrifugation (9).

Construction of a recombinant Charon 4A phage carrying the HSV-1 TK gene. A Charon 4A derivative carrying the HSV-1 TK gene and the minifibroin gene in pBR322 as a spacer was constructed as follows (see Fig. 1): pTK4miniFb DNA was partially digested with restriction endonuclease *Eco*RI, and one-cut, linear molecules were isolated by agarose gel electrophoresis with subsequent extraction with phenol and precipitation with ethanol. They were then ligated to Charon 4A arms (a gift from H. Okamoto) with T4 DNA ligase as described by Maniatis et al. (22). In vitro packaging of the recombinant DNA into phage particles was performed according to the protocol of Blattner et al. (1). The recombinant phages were screened by plating onto a lawn of *E. coli* K-12 Dp50SupF grown on X-gal (5-chloro-4-bromo-3-indolyl-β-D-galactoside) indicator plates (1). Ten individual clones that formed white plaques were isolated, and the restriction endonuclease cleavage patterns of DNA from the plaques were examined as described by Maniatis et al. (22). Four clones were obtained whose Charon 4A arms were connected to the *Eco*RI cut site located in the pBR322 region of pTK4miniFb. These clones were tested by the phage transfer method described below for activity to transform Ltk^- cells to the TK^+ phenotype, and all four were found to be competent for transformation. One of these clones, Ch4ATK2 (Fig. 1), was used for the gene transfer experiments described below.

The Ch4ATK2 phage clone was grown in liquid cultures according to the protocol of Blattner et al. and purified by polyethylene glycol-mediated phage precipitation, followed by CsCl centrifugation (43).

For the phage transfer method described below, purified phage were extensively dialyzed against SM (0.1 M NaCl–0.05 M Tris-hydrochloride [pH 7.5]–10 mM $MgSO_4$–0.001% gelatin) (22) and sterilized with chloroform. The chloroform was removed by low-speed centrifugation at 5 krpm for 20 min, and the phage were then incubated at 37°C for 1 h with gentle rotation. The phage was titrated on Dp50SupF bacteria and stored at 4°C in a sterile vial until used. Phage DNA was prepared as described by Maniatis et al. (22).

A gene library of human DNA in Charon 4A (15) was kindly provided by T. Maniatis.

Procedures for transformation and selection. (i) Phage transfer. Transformation of Ltk^- cells to the TK^+ phenotype was performed with the cloned HSV-1 TK gene contained in Ch4ATK2 recombinant phage particles. The procedure for transformation was based on the calcium phosphate method of Graham and van der Eb (8) as modified by Wigler et al. (40) and Lewis et al. (17). To improve the efficiency of transformation, we introduced further modifications which are described in detail below. The phage-mediated gene transfer method that we found most efficient was as follows.

Step 1. Preculture of recipient cells. Recipient Ltk^- cells in the logarithmic phase of growth in stock dishes were dissociated with EDTA-trypsin, plated at a cell density of 5×10^5 cells per 100-mm dish in Falcon dishes containing 10 ml of α-MEM supplemented with 10% calf serum, and incubated at 37°C under 5% CO_2 in air for 24 h.

Step 2. Dilution of phage. The Ch4ATK2 recombinant phage particles were gently dispersed at 25°C in 1 volume of 250 mM $CaCl_2$–0.9 mM Tris-hydrochloride (pH 7.9), prepared immediately before use from stock solutions of 2.5 M $CaCl_2$ and 1 mM Tris-hydrochloride (pH 7.9), in a sterile polycarbonate bottle with a round bottom.

Step 3. Coprecipitation of phage particles with calcium phosphate. *N*,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (50 mM, pH 6.87 at 25°C), containing 280 mM NaCl and 1.5 mM Na_2HPO_4, was prepared immediately before use from a stock solution of double this concentration and was sterilized by filtration through a Millex-GS filter (Millipore Corp.) after the pH was adjusted. One volume of the buffer was introduced dropwise down the side of the bottle containing the phage suspension. The mixture was maintained at 25°C for 10 min, during which time a fine precipitate developed.

Step 4. Adsorption. After formation of the microprecipitate of calcium phosphate-containing phage particles, 1 ml of the preparation was added by plastic pipette to the medium on recipient cells. The cells were maintained at 37°C under 5% CO_2 in air for 24 h.

Step 5. Expression. After the adsorption period, the medium was removed by aspiration, and the cells were washed once with 10 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.1), containing 137 mM NaCl and 3 mM KCl (HEPES buffered saline), fed again with 10 ml of fresh medium, and maintained at 37°C under 5% CO_2 in air for 40 h with one change of the medium.

Step 6. HAT selection. After the expression period, the medium was replaced by 10 ml of α-MEM supplemented with 10% calf serum and containing, per ml, 15 μg of hypoxanthine, 0.2 μg of aminopterin, 5 μg of thymidine, and 30 μg of glycine (HAT medium) to select cells with TK activity. The cells were maintained at 37°C under 5% CO_2 in air for 10 days with four changes of HAT medium. The medium was

renewed every day for the first 3 days, and then once every 3 days.

No colonies were obtained when over 10^8 untreated control Ltk$^-$ cells were plated in HAT medium.

(ii) DNA transfer. Transformation of Ltk$^-$ cells to the TK$^+$ phenotype with DNA of the cloned HSV-1 TK gene contained in recombinant plasmids or phage was performed under the same conditions as the phage-mediated gene transfer described above. Transformation efficiency comparable to that reported previously (12, 38, 40) could be attained by this method.

Colony count. After incubation for 10 days in HAT medium, the medium was removed, and the TK$^+$ colonies were fixed with 10% Formalin in phosphate-buffered saline containing 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4 for 30 min at room temperature and stained with an aqueous solution of 0.1% crystal violet for 30 min at room temperature. The colonies were then washed in running water, air-dried, and counted under a microscope.

Filter hybridization. High-molecular-weight DNA was isolated from cultured cells as described by Perucho et al. (30). The DNA was digested for 3 h with restriction endonucleases using 1 to 2 U of enzyme per μg of DNA. The products were subjected to electrophoresis in a 1.0% agarose gel, transferred to nitrocellulose filters, and hybridized as described by Southern with a slight modification (31). DNA used as probes was labeled with ^{32}P by nick translation (23). ^{32}P-labeled pBR322 DNA was kindly provided by K. Tokunaga.

Biohazards. The experiments were carried out according to the guidelines established by the Japanese Committee for Recombinant DNA Research.

RESULTS

Experimental design to establish a phage-mediated gene transfer method. To establish a method for phage-mediated gene transfer, we used a model system of HSV-1 TK gene and Ltk$^-$ cells because this system has been well characterized and is easily available (4, 12, 25, 36, 40). We first constructed a recombinant phage carrying cloned HSV-1 TK gene, which also carries the minifibroin gene cloned in pBR322 (Tsujimoto, unpublished data) as a spacer to accommodate the packaging of phage particles (Fig. 1), and introduced the phage particles into Ltk$^-$ cells by using the calcium phosphate technique of Graham and van der Eb (8). Successful gene transfer was assayed quantitatively by counting the number of TK$^+$ transformant colonies developed after HAT selection (18).

Parameters governing phage-mediated gene transfer. To improve the efficiency of gene transfer, we examined the following parameters governing phage-mediated gene transfer. Unless otherwise stated, gene transfer was performed according to the standard protocol described above, using a phage dose of 3×10^7 plaque-forming units (PFU) per 100-mm dish.

(i) Preculture of recipient cells. (a) Cell density. When the cells were inoculated 24 h before phage adsorption, the number of TK$^+$ transformant colonies increased linearly with increases in the initial cell density and reached a peak at a cell density of about 5×10^5 cells per 100-mm dish (Fig. 2). Further increases in the cell density resulted in a gradual decrease in the number of TK$^+$ colonies. At lower cell densities, cells were apparently damaged by the phage adsorption treatment, with resultant low efficiencies of gene transfer.

(b) Culture media. When the competence of α-MEM to sustain gene transfer was defined as 100%, that of F-12, Eagle MEM, and Dulbecco modified MEM was 21, 89, and 94%, respectively.

(ii) Coprecipitation of phage particles with calcium phosphate. (a) pH and buffer. As reported for DNA-mediated gene transfer (8), one of the most important parameters was the pH of the buffer used for coprecipitation of phage particles with calcium phosphate. Figure 3 shows that high efficiency of phage-mediated gene transfer was achieved in only a narrow range of pH 6.80 to 6.90 at 25°C. This pH optimum is slightly more acidic than that for DNA-mediated gene transfer (8). In this pH range, only a very fine precipitate developed, and the preparation damaged the recipient cells only slightly during an adsorption period of 24 h.

The effect of buffer systems having pK_a values around this pH optimum was also examined at pH 6.85 (25°C). When the competence of BES buffer to sustain gene transfer was defined as 100%, that of piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), and HEPES buffers was 100, 94, and 70%, respectively.

We used BES buffer (pH 6.87 at 25°C) in further studies. Because of the importance of the pH of the buffer, we adjusted the pH very carefully, by incubating stock solution at 25°C in a water bath, after thermal equilibrium was attained, and we sterilized the buffer by filtration through a Millex-GS filter (Millipore). Coprecipitation of phage particles with calcium phosphate was also carried out at 25°C in a water bath.

(b) Period of coprecipitation. When calcium solution containing phage particles and BES buffer containing inorganic phosphate were mixed, a fine precipitate rapidly developed and gradually became coarser on further incubation. Figure 4A shows the effect of the incubation time on phage-mediated gene transfer. The optimal incubation time was 7 to 20 min; further incubation resulted in a gradual decrease in gene transfer efficiency.

(c) Calcium phosphate. In the absence of calcium phosphate, application of 3×10^7 phage particles resulted in about one TK$^+$ transfor-

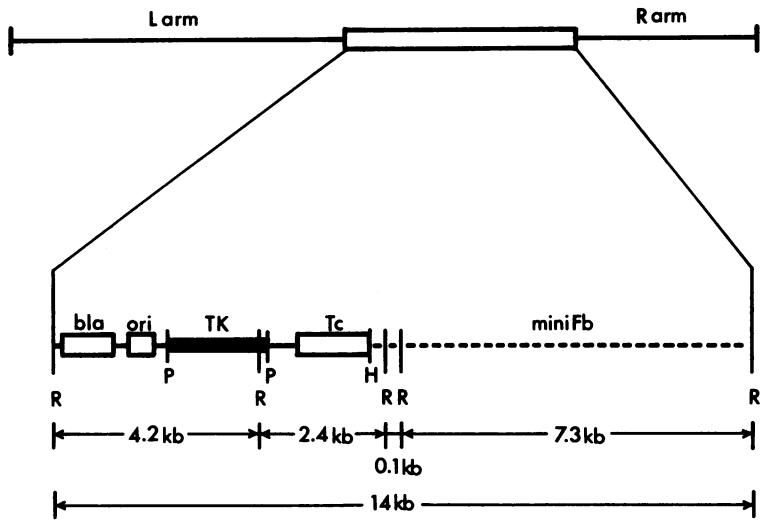

FIG. 1. Restriction map of Ch4ATK2 recombinant phage. Symbols: Solid lines, pBR322 sequences; thick solid line, HSV-1 TK gene in a 2.0-kb *Pvu*II segment; dotted line, minifibroin gene in a 7.6-kb *Eco*RI/*Hin*dIII segment; bla, ampicillin resistance gene; ori, origin of replication of pBR322; Tc, tetracycline resistance gene. R, P, and H refer to restriction endonuclease sites for *Eco*RI, *Pvu*II, and *Hin*dIII, respectively.

mant per dish. Inclusion of calcium phosphate as an adjuvant enhanced phage-mediated gene transfer at least 250-fold (data not shown).

(iii) Adsorption of phage particles. (a) Volume of calcium phosphate suspension. Addition of various volumes of calcium phosphate suspension, containing a constant amount of phage particles, to 10 ml of medium on recipient cells showed that the optimum volume for gene transfer was about 1 ml (data not shown).

(b) Adsorption time. The number of TK^+ transformant colonies increased linearly with the time of adsorption and reached a plateau at about 18 h (Fig. 4B). The optimum time for adsorption of phage particles was 18 to 30 h. Further increase in the adsorption time resulted in a rather rapid decrease in the number of TK^+ colonies.

(c) Treatment with dimethyl sulfoxide, glycerol, and sucrose. Several groups have reported that treatment of recipient cells with dimethyl sulfoxide, glycerol, or sucrose at the end of the adsorption period greatly enhances transformation by either DNA or chromosomes (6, 17, 19, 26, 34). We examined the effects of dimethyl sulfoxide, glycerol, and sucrose under various conditions and found that these treatments did not significantly affect the number of TK^+ transformant colonies, although larger colonies developed when the recipient cells were treated with these reagents (data not shown).

(iv) Expression time. The expression time required for the HSV-1 TK gene, introduced by phage-mediated gene transfer, was essentially the same as reported for chromosome-mediated gene transfer (17). Thus, we used an expression time of 40 h.

Linear relationship between phage dose and number of transformants. To evaluate the effect of phage-mediated gene transfer, it is very important to determine the relation between the phage dose and number of transformants and to determine the phage dose that yields the highest number of transformants.

Figure 5 shows the results. The number of TK^+ transformant colonies increased linearly with increases in the phage dose, up to at least 5×10^7 PFU per dish. From the linear relationship, we calculated the efficiency of phage-mediated gene transfer to be 1.0×10^{-5} ($1\ TK^+$ transformant per 10^5 phage particles per 10^6 cells). This value is about 30 to 60 times higher than the values determined for DNA-mediated gene transfer of the cloned HSV-1 TK gene (data not shown).

To characterize the phage dosage effect further, we determined the efficiency of phage-mediated gene transfer in the presence of various amounts of carrier phage particles which lack the HSV-1 TK gene, using the Charon 4A library phage for human DNA as a carrier (Table 1). The number of TK^+ transformant colonies was almost constant in spite of increases in the dose of carrier phage up to 10^9 PFU per dish. Further increases of carrier phage resulted in a decrease of TK^+ colonies, the value being 41% of that without the carrier at a dose of 10^{10} PFU per dish and 2% at a dose of 10^{11} PFU per dish.

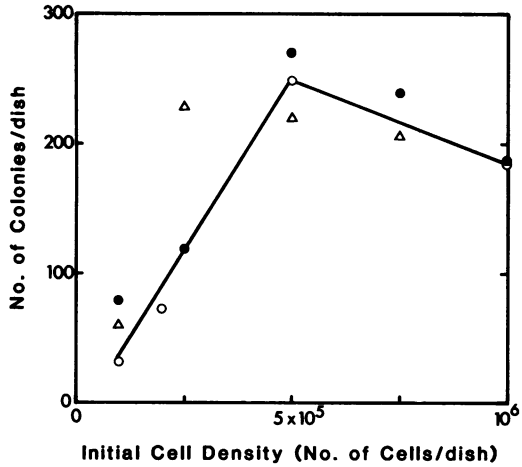

FIG. 2. Effect of the initial cell density of preculture on phage-mediated gene transfer. The Ltk$^-$ cells were inoculated into 10 ml of α-MEM in 100-mm dishes at the various initial cell densities indicated, 24 h before application of phage particles. One milliliter of Ch4ATK2 recombinant phage particles (3×10^7 PFU/ml), coprecipitated with calcium phosphate at 25°C for 10 min in 25 mM BES buffer (the initial pH of BES buffer was 6.87 at 25°C), was applied to the recipient cells. After an adsorption period of 24 h at 37°C under 5% CO_2 in air, the cells were washed once with 10 ml of HEPES buffered saline, refed with 10 ml of α-MEM, and maintained at 37°C under 5% CO_2 in air for 40 h for expression of the HSV-1 TK gene. After the expression period, the medium was replaced by HAT medium, and the cells were further maintained at 37°C under 5% CO_2 in air for 10 days with four changes of HAT medium to select TK$^+$ transformants. After HAT selection, the TK$^+$ colonies were fixed with 10% Formalin and stained with 0.1% crystal violet, and numbers of TK$^+$ colonies were determined under a microscope. The results of three separate experiments (○, ●, △) are shown. Each point represents the average of three dishes.

However, increases in the initial cell density of preculture partially overcame the toxic effect of high dosage of carrier phage, and using an initial cell density of 1.0×10^6 cells per dish, we attained an efficiency of 1.0×10^{-5} for gene transfer also in the presence of 10^{10} PFU of carrier phage per dish (data not shown).

The procedure for phage-mediated gene transfer described here is applicable to different kinds of cells. With a rat fibroblast cell line, F2408tk$^-$ cells, as recipient cells, we obtained essentially the same results (Table 2). Because this cell line was more sensitive to the application of phage particles, we increased the initial cell density of preculture to 1.0×10^6 cells per dish.

HSV-1 TK gene and pBR322 sequences in transformant DNA. To analyze the exogenously introduced DNA sequences by phage-mediated gene transfer, we performed blot hybridization experiments. Ltk$^-$ cells were transformed with Ch4ATK2 recombinant phage particles (10^6 PFU per dish), and nine individual TK$^+$ transformant colonies were isolated and grown in mass culture under HAT selection. High-molecular-weight DNA was isolated from each transformant, completely digested with restriction endonuclease *Eco*RI, subjected to electrophoresis on 1% agarose, transferred to nitrocellulose filters, and analyzed by blot hybridization.

As seen from Fig. 1, complete digestion of Ch4ATK2 DNA with *Eco*RI produced Charon 4A arms and the following four fragments: a 4.2-kb fragment carrying the ampicillin resistance gene, the origin of replication of pBR322, and the HSV-1 TK gene; a 2.4-kb fragment carrying the tetracycline resistance (Tc^r) gene and part of the minifibroin sequences; and a 0.1-kb fragment and a 7.3-kb fragment, which were derived from minifibroin sequences.

Blot analysis of the HSV-1 TK gene sequences in the transformant DNA, using purified HSV-1 TK gene as a probe (Fig. 6), showed that all nine transformants contained TK sequences only in a 4.2-kb fragment. This suggests

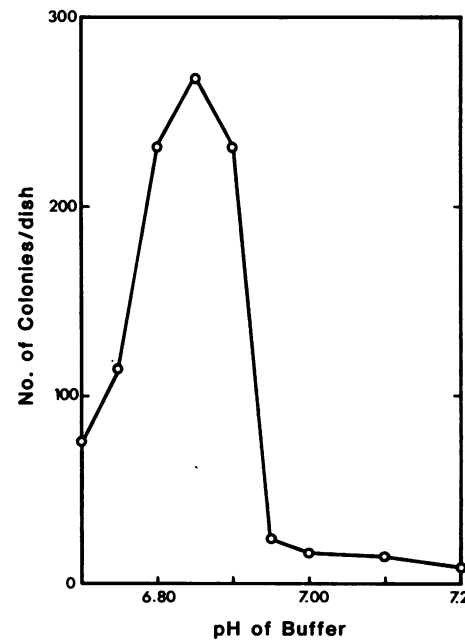

FIG. 3. Effect of pH of the buffer for coprecipitation of phage particles with calcium phosphate on phage-mediated gene transfer. Ch4ATK2 phage particles were coprecipitated with calcium phosphate in 25 mM PIPES buffer at the various pH values indicated. The pH values indicated are the initial values for the buffer. The initial cell density of preculture was 5×10^5 cells per dish. Other conditions were as described for Fig. 2.

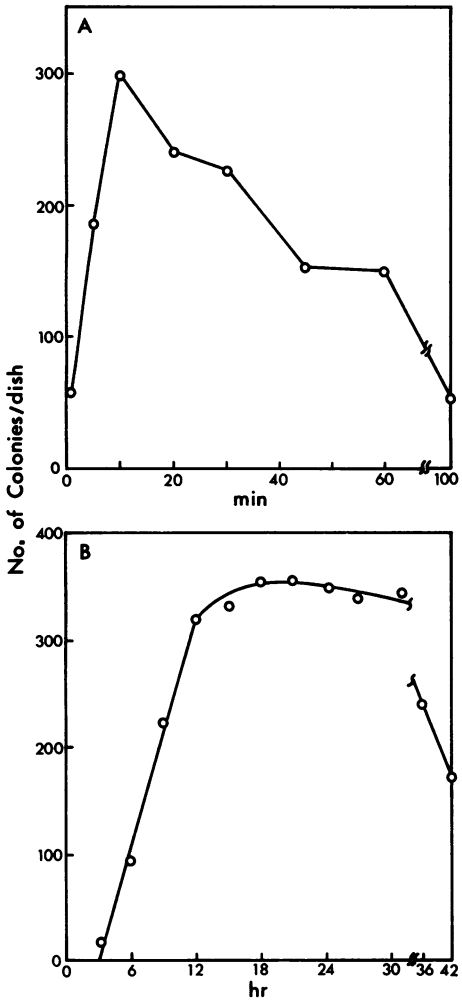

FIG. 4. Effects of the incubation periods for coprecipitation of phage particles with calcium phosphate and for adsorption of phage particles to Ltk$^-$ cells on phage-mediated gene transfer. (A) Coprecipitation time: Ch4ATK2 phage particles were coprecipitated with calcium phosphate for the periods indicated. (B) Adsorption time: The phage particles coprecipitated with calcium phosphate were applied to Ltk$^-$ cells, and the cells were maintained at 37°C under 5% CO_2 in air for the periods indicated. The initial cell density of preculture was 5×10^5 cells per dish. Other conditions were as described for Fig. 2.

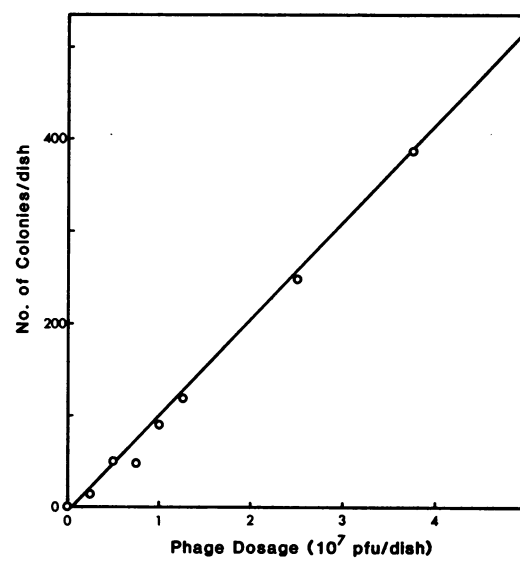

FIG. 5. Linear relation between phage dosage and number of TK$^+$ transformants. Various titers of Ch4ATK2 phage particles coprecipitated with calcium phosphate were applied to Ltk$^-$ cells. Other conditions were as described for Fig. 2.

that the DNA sequences from the junction between the left arm of Charon 4A and the ampicillin resistance gene to and the HSV-1 TK gene (see Fig. 1) were stably conserved without rearrangement in all nine transformants. This observation is further supported by the results of blot analysis of pBR322 sequences (data not shown) showing that all nine transformants contained pBR322 sequences in this 4.2-kb fragment.

However, some rearrangement of pBR322 sequences occurred in the transformants, because some transformants contained pBR322 sequences in a 7- to 8-kb fragment in addition to the 4.2-kb fragment. Furthermore, no 2.4-kb fragment carrying the Tc^r gene was detected in any of the nine transformants. This indicates that a 2.4-kb fragment carrying the Tc^r gene was lost or translocated somewhere in all the transformants examined.

Because there was extensive cross-hybridiza-

TABLE 1. Effect of carrier phage dosage on phage-mediated gene transfer[a]

Dosage of carrier phage (PFU per dish)	TK$^+$ colonies per dish[b]
0	258, 410, — (334)
10^8	288, 324, 332 (315)
10^9	344, 344, 396 (361)
10^{10}	126, 132, 152 (137)
10^{11}	0, 4, 14 (6)

[a] Ch4ATK2 phage particles (3×10^7 PFU per dish) with various titers of Charon 4A library phage for human DNA were coprecipitated with calcium phosphate. The initial cell density of preculture was 5×10^5 cells per dish. Other conditions were as described for Fig. 2.

[b] Three dishes; parentheses indicate average. —, Contaminated.

TABLE 2. Phage-mediated gene transfer to rat F2408tk⁻ cells[a]

Initial cell density of preculture (cells per dish)	TK⁺ colonies per dish[b] after Ch4ATK2 added:	
	10^5 PFU	5×10^6 PFU
5.0×10^5	0, 0, 0 (0)	—
7.5×10^5	0, 1, 2 (1)	—
1.0×10^6	1, 1, 1 (1)	18, 21, 30 (23)
2.0×10^6	0, 2, 2 (1)	—
3.0×10^6	0, 0, 0 (0)	—

[a] In place of Ltk⁻ cells, rat F2408tk⁻ cells were used as recipient cells. Other conditions were as described for Fig. 2.

[b] Three dishes; parentheses indicate average. —, Not determined.

tion of minifibroin sequences to Ltk⁻ cell DNA (data not shown), it was not possible to analyze minifibroin sequences in the transformant DNA.

DISCUSSION

The phage-mediated gene transfer described in this publication has at least two advantages over DNA-mediated gene transfer. First, a high efficiency of gene transfer, 1 transformant per 10^5 phage particles per 10^6 cells, was attained. This efficiency is one to two orders higher than the values reported for DNA-mediated gene transfer, although it is not as high as the highest values reported for chromosome-mediated gene transfer (see Table 3). Second, a high efficiency of phage-mediated gene transfer can be attained without carrier DNA. This excludes the complication of effects of carrier DNA on integration of exogenous DNA sequences into the host genome and their subsequent expression (30).

Our blot analyses (Fig. 6) showed that the left flanking sequences of the selected TK gene were highly conserved, although a region near the end of the Tc^r gene in pBR322 was rearranged after being introduced into cultured mouse cells. This rearrangement of the right flanking sequences of the TK gene was probably due to the presence of incompatible sequences in the recombinant phage used here. Recently, the Tc^r gene or its flanking sequences in pBR322 or both have been found to be incompatible with cultured mammalian cells (14, 21, 28). The incompatible sequences described by Kretshmer et al. (13) are situated near the end of the Tc^r gene, probably between the *Ava*I cut site and the *Sal*I cut site, whereas the "poison" sequences described by Lusky and Botchan (21) occur between the *Pvu*II cut site and the origin of the replication. If such incompatible sequences are not present in the insert of a recombinant phage, the conserved region of the flanking sequences of a selected gene may be much wider.

These observations open up possibilities of various applications of phage-mediated gene transfer. First, phage-mediated gene transfer should be useful in experiments on gene expression of cloned DNA sequences in cultured mammalian cells, because introduction of an appropriate gene with its long flanking sequences can be achieved. The flanking sequences are thought to have an important role in the control of expression of the gene. Recombinant phage DNA carrying an appropriate gene with its flanking sequences has been introduced into cells to study the expression of a cloned gene in cultured mammalian cells (11, 14). However, our observations suggest that in place of recombinant phage DNA, phage particles can be introduced more easily into the recipient cells for this purpose.

Second, using a gene library of mammalian DNA in lambda phage vectors, phage-mediated gene transfer can probably be used in isolation of a selectable marker gene from mammalian DNA. This approach has several advantages, as follows. (i) The procedure for construction of the gene library of mammalian DNA in lambda phage vectors has been established (1, 10, 16, 22, 33). Recombinant phage particles covering the gene library, once constructed, can easily be amplified in large amounts in *E. coli*. (ii) For rescue of a mammalian selectable marker gene in *E. coli*, bacterial marker sequences which are functional in *E. coli* and compatible with cultured mammalian cells, such as the ampicillin resistance gene, the origin of replication of pBR322 (29, 41), and suppressor F sequences, etc., can be introduced onto the arms of lambda vectors before construction of the gene library.

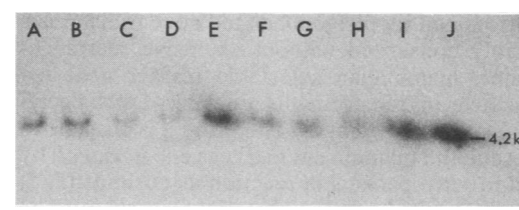

FIG. 6. Hybridization profile of HSV-1 TK sequences in TK⁺ transformant DNA. Ltk⁻ cells were transformed with Ch4ATK2 phage particles (10^6 PFU per dish) coprecipitated with calcium phosphate, and nine individual TK⁺ transformant colonies were isolated. High-molecular-weight DNA (10 to 20 μg) extracted from each transformant (lanes A to I) and pTK4miniFb plasmid DNA (lane J) were cleaved with *Eco*RI and analyzed by filter hybridization after electrophoresis on a 1.0% gel, using nick-translated 3.4-kb *Bam*HI fragments carrying HSV-1 TK gene (10^8 cpm/μg) as a probe. The molecular size of the pTK4miniFb *Eco*RI fragments is indicated.

TABLE 3. Efficiency of gene transfer

Method of gene transfer	Genetic material	Selected genes coding for:	Original data[a]	Efficiency[b]	Applicable gene dose[c]	Frequency of transformants[d]	Reference
DNA-mediated	3.4-kb *Bam*HI fragments carrying HSV-1 TK gene	TK	1/40 pg	8.5×10^{-8}			(40)
	pBR322 carrying HSV-1 TK gene	TK	121/10 ng	7.6×10^{-8}			(12)
	Genomic DNA from hamster, human, and mouse	TK APRT	10/20 μg	1.5×10^{-6}	6.7×10^{6}	1.0×10^{-5}	(38, 39)
	Genomic DNA from CHO cells	TK	43/20 μg	6.4×10^{-6}	6.7×10^{6}	4.3×10^{-5}	(17)
		Mtx[re]	20/20 μg	3.0×10^{-6}	6.7×10^{6}	2.0×10^{-5}	
Chromosome-mediated	Chromosomes from CHO cells	Mtx[r]	700/2 × 10^{6} cell eq.[f]	3.5×10^{-4}	2.0×10^{6}	7.0×10^{-4}	(17)
Phage-mediated	Charon 4A phage carrying CHO APRT gene	APRT	24/2 × 10^{7} PFU	1.2×10^{-6}	2.5×10^{2}[g]	3.0×10^{-10}[h]	(20)
	Charon 4A phage carrying HSV-1 TK gene	TK		1.0×10^{-5}	1.3×10^{3} to 1.3×10^{4}[g]	1.3×10^{-8} to 1.3×10^{-7}[h]	This work

[a] Number of transformants/amount of genetic material added per 10^6 recipient cells.
[b] Number of transformants per gene equivalent or haploid genome equivalent. The values were calculated by taking the mammalian haploid genome size as 1.8×10^{12} daltons.
[c] Haploid genome equivalent per 10^6 recipient cells.
[d] Number of transformants per recipient cell.
[e] Methotrexate resistance.
[f] Cell equivalent.
[g] The number of recombinant phages required for a complete mammalian DNA library is calculated as 8×10^5, using 20 kb as the average length of the inserted DNA and 3×10^9 base pairs as the mammalian genome size.
[h] Calculated values (efficiency × applicable gene dose × 10^{-6}).

Use of modified vectors for construction of the gene library guarantees that all inserted sequences derived from mammalian DNA are always flanked with bacterial marker sequences active in *E. coli*. (iii) If the DNA sequences introduced by phage-mediated gene transfer are stably conserved without extensive rearrangement, mammalian selectable marker gene sequences in transformants are flanked with bacterial marker sequences. Therefore it is possible to rescue the mammalian marker gene in *E. coli* by an in vitro packaging reaction as cosmids (5, 7) or as phages using the bacterial marker. (iv) The high efficiency of phage-mediated gene transfer makes it possible to transform mutant cells with recombinant phage particles covering the gene library. The number of recombinant phages required for a complete mammalian DNA library is calculated as 8×10^5, using 20 kb as the average length of the inserted DNA and 3×10^9 base pairs as the mammalian genome size (15). If we extrapolate the efficiency of phage-mediated gene transfer that we observed for the transfer of the HSV-1 TK gene, we can expect the transfer of a specific gene once per 8×10^{10} PFU of the library phage. In fact, using a gene library of human DNA in Charon 4A and Ltk$^-$ cells, we have observed transfer of the human TK gene once per 2.8×10^{10} PFU of the library phage (Ishiura et al., manuscript in preparation).

In spite of these advantages, the procedure for phage-mediated gene transfer should be refined, because application of over 10^{10} PFU of phage particles per dish is toxic to cultured mammalian cells, resulting in a greatly reduced efficiency of gene transfer (Tables 1 and 3). Improvement in efficiency of gene transfer or an applicable phage dose, or both, should be attained for isolation of single-copy genes by phage-mediated gene transfer.

Recently, Hamada et al. succeeded in rescuing the HSV-1 TK gene in *E. coli* from one of the transformants described in this publication, by using an ampicillin resistance gene and the origin of pBR322 as selection markers (Hamada et al., manuscript in preparation). Thus, pBR322 sequences carrying the ampicillin resistance gene, the origin of replication, and the HSV-1 TK gene, previously introduced into the host cells by phage-mediated gene transfer, are stably con-

served physically and also functionally in the cells.

Lowy et al. reported transformation of Laprt$^-$ cells to the APRT$^+$ phenotype with recombinant phage particles carrying the Chinese hamster ovary cell (CHO) APRT gene, but the efficiency of the transformation was one order less than that which we have attained (20).

ACKNOWLEDGMENTS

We are grateful to T. Maniatis, A. Hakura, Y. Tsujimoto, H. Okamoto, K. Tokunaga, and E. Ishikawa for kind gifts of a gene library, a rat cell line, a plasmid, the arms of Charon 4A, DNA size markers, and ^{32}P-labeled DNA, respectively. M.I. also thanks H. Okamoto, Y. Tsujimoto, K. Tokunaga, and T. Ogino for information on in vitro packaging of phage particles, preparation of plasmid DNA, Southern blotting, and HAT selection, respectively, and A. T. Ishida and N. Shimizu, respectively, for reading and typing the manuscript.

ADDENDUM IN PROOF

All seven transformants examined were stable in nonselective medium for at least 50 days.

LITERATURE CITED

1. Blattner, F. R., B. G. Williams, A. E. Blechl, K. D. Thompson, H. E. Faber, L. A. Furlong, D. J. Grunwald, D. O. Kiefer, D. D. Moore, J. W. Schumm, E. L. Sheldon, and O. Smithies. 1977. Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. Science 196:161–169.
2. Burch, J. W., and O. W. McBride. 1975. Human gene expression in rodent cells after uptake of isolated metaphase chromosomes. Proc. Natl. Acad. Sci. U.S.A. 72:1797–1801.
3. Clewell, D. B. 1972. Nature of Col E_1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. 110:667–676.
4. Colbera-Garapin, F., S. Chousterman, F. Horodniceanu, P. Kourilsky, and A.-C. Garapin. 1979. Cloning of the active thymidine kinase gene of herpes simplex virus type 1 in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. 76:3755–3759.
5. Colins, J., and B. Hohn. 1978. Cosmids: a type of plasmid gene-cloning vector that is packageable *in vitro* in bacteriophage λ heads. Proc. Natl. Acad. Sci. U.S.A. 75:4242–4246.
6. Copeland, N. G., and G. M. Cooper. 1979. Transfection of exogenous and endogenous murine retrovirus DNA. Cell 16:347–356.
7. Fukumaki, Y., K. Shimada, and Y. Takagi. 1976. Specialized transduction of colicin E1 DNA in *Escherichia coli* K-12 by phage lambda. Proc. Natl. Acad. Sci. U.S.A. 73:3238–3242.
8. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–461.
9. Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064–1066.
10. Hohn, B., and K. Murray. 1977. Packaging recombinant DNA molecules into bacteriophage particles *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 74:3259–3263
11. Hynes, N. E., N. Kennedy, U. Rahmsdorf, and B. Groner. 1981. Hormone-responsive expression of an endogenous proviral gene of mouse mammary tumor virus after molecular cloning and gene transfer into cultured cells. Proc. Natl. Acad. Sci. U.S.A. 78:2038–2042.
12. Kit, S., H. Otsuka, H. Qavi, D. Trkula, D. R. Dubbs, and M. Hazen. 1980. Biochemical transformation of thymidine kinase (TK)-deficient mouse cells by herpes simplex virus type 1 DNA fragments purified from hybrid plasmids. Nucleic Acids Res. 8:5233–5253.
13. Kretschmer, P., A. H. Bowman, M. H. Huberman, L. Sander-Haigh, L. Killos, and W. F. Anderson. 1981. Recovery of recombinant bacterial plasmids from *E. coli* transformed with DNA from microinjected mouse cells. Nucleic Acids Res. 9:6199–6217.
14. Kurtz, D. 1981. Hormonal inducibility of rat α_{2u}-globulin genes in transfected mouse cells. Nature (London) 291:629–631.
15. Lawn, R. M., E. F. Fritsch, R. C. Parker, G. Blake, and T. Maniatis. 1978. The isolation and characterization of linked δ- and β-globin genes from a cloned library of human DNA. Cell 15:1157–1174.
16. Leder, P., D. Tiemeir, and L. Enquist. 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the λgtWES system. Science 196:175–177.
17. Lewis, W. H., P. R. Srinivasan, N. Stokoe, and L. Siminovitch. 1980. Parameters governing the transfer of the genes for thymidine kinase and dihydrofolate reductase into mouse cells using metaphase chromosomes or DNA. Somatic Cell Genet. 6:333–347.
18. Littlefield, J. W. 1965. Studies on thymidine kinase in cultured mouse fibroblasts. Biochim. Biophys. Acta 95:14–22.
19. Lowy, D. R., E. Rands, and E. M. Scolnick. 1978. Helper-independent transformation by unintegrated Harvey sarcoma virus DNA. J. Virol. 26:291–298.
20. Lowy, I., A. Pellicer, J. F. Jackson, G.-K. Sim, S. Silverstein, and R. Axel. 1980. Isolation of transforming DNA: cloning the hamster aprt gene. Cell 22:817–823.
21. Lusky, M., and M. Botchan. 1981. Inhibition of replication in simian cells by specific pBR322 DNA sequences. Nature (London) 293:79–81.
22. Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connel, D. Quon, G. K. Sim, and A. Efstratiadis. 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell 15:687–701.
23. Maniatis, T., A. Jeffrey, and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. 72:1184–1188.
24. McBride, O. W., and H. L. Ozer. 1973. Transfer of genetic information by purified metaphase chromosomes. Proc. Natl. Acad. Sci. U.S.A. 70:1258–1262.
25. McKnight, S. L. 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. 8:5949–5964.
26. Miller, C. L., and F. H. Ruddle. 1978. Co-transfer of human X-linked markers into murine somatic cells via isolated metaphase chromosomes. Proc. Natl. Acad. Sci. U.S.A. 75:3346–3350.
27. Murayama-Okabayashi, F., Y. Okada, and T. Tachibana. 1971. A series of hybrid cells containing different ratios of parental chromosomes formed by two steps of artificial fusion. Proc. Natl. Acad. Sci. U.S.A. 68:38–42.
28. Myers, R., and R. Tjian. 1980. Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. Proc. Natl. Acad. Sci. U.S.A. 77:6491–6495.
29. Perucho, M., D. Hanahan, L. Lipsich, and M. Wigler. 1980. Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature (London) 285:207–210.
30. Perucho, M., D. Hanahan, and M. Wigler. 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell 22:309–317.
31. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
32. Stanners, C. P., G. Eliceiri, and H. Green. 1971. Two types of ribosome in mouse-hamster hybrid cells. Nature (London) New Biol. 230:52–53.
33. Sternberg, N., D. Tiemeier, and L. Enquist. 1977. In vitro packaging of a λ Dam vector containing EcoR I DNA

fragments of *Escherichia coli* and phage P1. Gene 1:255–280.

34. **Stowe, N. D., and N. M. Wilkie.** 1976. An improved technique for obtaining enhanced infectivity with herpes simplex type 1 DNA. J. Gen. Virol. 33:447–458.
35. **Tsujimoto, Y., and Y. Suzuki.** 1979. Structural analysis of the fibroin gene at the 5′ end and its surrounding regions. Cell 16:425–436.
36. **Wagner, M. J., J. A. Sharp, and W. G. Summers.** 1981. Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. Proc. Natl. Acad. Sci. U.S.A. 78:1441–1445.
37. **Weiss, B., A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, and C. C. Richardson.** 1968. Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. J. Biol. Chem. 243:4543–4555.
38. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell 14:725–731.
39. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 76:1373–1376.
40. **Wigler, M., S. Silverstein, S.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223–232.
41. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell 16:777–785.
42. **Willecke, K., and F. Ruddle.** 1975. Transfer of the human gene for hypoxanthine-guanine phosphoribosyltransferase via isolated human metaphase chromosomes into mouse L-cells. Proc. Natl. Acad. Sci. U.S.A. 72:1792–1796.
43. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and C. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology 40:734–744.